Appendix B

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

Gene 189 (1997) 269–275

Myelin and lymphocyte protein (MAL/MVP17/VIP17) and plasmolipin are members of an extended gene family

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Received 3 September 1996; revised 12 November 1996; accepted 12 November 1996; Received by J.A. Engler

Abstract

An increasing number of four-transmembrane proteins has been found to be associated with CNS and PNS myelin. Some of these proteins play crucial roles in the development and maintenance of the nervous system. In the CNS, proteolipid protein (PLP) is mutated in the myelin disorder Pelizaeus-Merzbacher disease and in spastic paraplegia, while in the PNS, peripheral myelin protein 22 (PMP22) and connexin32 (C × 32) are culprit genes in the most frequent forms of hereditary peripheral neuropathies. Myelin and lymphocyte protein (MAL; also called MVP17 or VIP17) and plasmolipin are additional tetraspan proteins that are highly expressed by myelinating glial cells. However, little is known about the role of these proteins in the nervous system. As a prerequisite for functional genetic approaches in the mouse, we have isolated and characterized a mouse MAL cDNA and the corresponding structural MAL gene. Computer-aided analysis and database searches revealed that MAL belongs to a larger gene family which also includes plasmolipin, BENE and the expressed sequence tag (EST) H09290. While the overall amino acid sequence identities between mouse MAL and the related proteins are relatively low (29–37%), the conserved motif -[Q/Y-G-W-V-M-F/Y-V]- which is found at the junction of the first extracellular loop and the second membrane-associated domain serves as a fingerprint for the MAL protein family. Expression analysis of the members of the MAL gene family indicates widespread expression in various tissues, suggesting a common role of these proteins in cell biology. © 1997 Elsevier Science B.V.

Keywords: Nervous system; Schwann cell; Oligodendrocyte; Tetraspan protein; BENE; Gene structure

1. Introduction

The highly hydrophobic proteolipid protein MAL has first been identified as a marker of human T cell maturation (Alonso and Weissman, 1987) and, based on the extensive alternative splicing of its mRNA, human MAL (hMAL) is thought to exist in multiple isoforms (Rancano et al., 1994a,b). The rat homologue rMAL was isolated by two different screening approaches which were aimed at the identification of either genes specifically expressed by myelinating oligodendrocytes (Schaeren-Wiemers et al., 1995a,b), or proteins which might play a role in intracellular sorting and transport processes during myelination (Kim et al., 1995). Furthermore, the canine homologue cMAL has been described as a component of immuno-isolated transport vesicles from Madin-Darby canine kidney (MDCK) cells (Zacchetti et al., 1995). Based on these results, it was hypothesized that MAL might be involved in vesicular trafficking and cycling between the Golgi complex and the apical plasma membrane.

Northern blot analysis of rMAL mRNA revealed a restricted expression pattern in spleen, kidney, spinal cord, brain and peripheral nerves (sciatic nerve) with the highest mRNA levels found in neural tissues, tightly correlated with CNS myelination (Kim et al., 1995; Schaeren-Wiemers et al., 1995b). Complementary in situ hybridization and immunohistochemical analyses confirmed that in the CNS, rMAL is mainly expressed by

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Abbreviations: aa, amino acid(s); bp, base pair(s); kb, kilobase(s) or 1000 bp; MAL, myelin and lymphocyte protein.

PII S0378-1119(96)00861-X

myelinating oligodendrocytes, but even stronger expression was found in myelinating Schwann cells in the PNS (Schaeren-Wiemers et al., 1995b).

Recently, the myelin-associated proteolipid protein plasmolipin which shares some structural similarities with MAL has been identified and characterized by molecular cloning in the rat (Fischer and Sapirstein, 1994; Gillen et al., 1996). Similar to rMAL, plasmolipin mRNA expression appears to be restricted to kidney and myelinating glial cells of the CNS and PNS. Furthermore, the regulation of plasmolipin mRNA expression during neural development and sciatic nerve regeneration showed a tight correlation with myelination (Gillen et al., 1996). In agreement with these results, plasmolipin has been described as a component of both CNS and PNS myelin. Furthermore, plasmolipin has been localized to the apical surface of tubular epithelial cells in the kidney (Cochary et al., 1990; Fischer et al., 1994). The addition of a crude preparation of purified plasmolipin to lipid bilayers was able to induce the formation of voltage-dependent and potassium-selective ion channels in vitro (Tosteson and Sapirstein, 1981), but the exact functional role of this intriguing protein in neural and non-neural tissues remains to be clarified.

MAL and plasmolipin join a growing group of tetraspan membrane proteins which are associated with myelin. In particular, various mutations affecting the gene encoding the major protein component of CNS myelin, PLP, lead to severe CNS myelin deficiencies in inherited myelin disorders (reviewed by Nave and Boespflug-Tanguy, 1996). In the PNS, the peripheral myelin protein PMP22 gene is mutated in the most common forms of hereditary motor and sensory neuropathies (Charcot-Marie-Tooth disease type 1A (CMT1A); reviewed by Suter and Snipes, 1995a,b), and the gap junction component C×32 has been found to be mutated in X-linked forms of Charcot-Marie-Tooth disease (CMTX; reviewed by Spray and Dermietzel, 1995).

Naturally occurring mutations in animals and humans as well as artificially generated mutations in rodents have improved considerably our knowledge about the crucial role of putative four-transmembrane proteins in neural development, in the maintenance of the normal organism, and in various disease processes (Adlkofer et al., 1995; Suter and Snipes, 1995a; Magyar et al., 1996; Nave and Boespflug-Tanguy, 1996; Sereda et al., 1996). Since the mouse has become the most versatile animal model for the generation of targeted myelin mutants (reviewed by Gu et al., 1994; Kuhn et al., 1995; Soriano, 1995), we have isolated a mouse MAL (mMAL) cDNA and the corresponding genomic mMAL gene as a prerequisite for further studies. The analysis of the obtained sequences revealed that MAL belongs to a distinct subfamily of tetraspan proteins, including plasmolipin, which are widely expressed in various tissues.

2. Material and methods

2.1. Cloning and nucleic acid analysis

Standard methods were used according to Sambrook et al. (1989). A [^{32}P]dATP (Amersham) labeled rMAL cDNA (random-primed DNA labeling kit; Pharmacia) was used to screen a cDNA library (λZAP; Stratagene) from the brain of 8-day-old mice. Positive clones were converted to pBluescript according to the manufacturer's recommendations and sequenced (Sequenase kit, USB) using the following oligonucleotide primers: MALe1f: CGTGTCCAGTCCCAAG; MALe1r: ACTCACAAACGAAGAGC, MALe2f: TCTTTGGAGGCCTGGTG; MALe2r: GTGTGATCCAGGAAGTC; MALe3f: GATGCAGCCTACCACTG; MALe3r: GCGGCGATGTTTTCATG; MALe4r: GCTGACCAGTTAATTGC; MALe4.1: CCAGACTATTCACAAAG; MALe4.2: AGAGACGCTATCCTGGTG; MALe4.3: GTGGGGACAAAGTGAGA; MALe4.4: GCTTGTGTTTTAGTATG.

The isolated mMAL cDNA was used to screen a 129Sv mouse genomic pTCF cosmid library (Pan et al., 1994). Identified clones were analyzed by restriction mapping and Southern blot analysis as well as partial sequencing (using the MAL internal primers and the primers pTCFupper: CCTCAACCTACTACTGG and pTCFlower: AGTGCGGCGACGATAGT). For the determination of the intron lengths using the above exon specific primers, PCR analysis was performed either according to Kogan et al. (1987), or using the long-range GeneAmp XL PCR kit (Perkin-Elmer). Conditions for hot-start PCR reactions (in the presence of AmpliVax beads) were 2 min initial denaturation, followed by 40 cycles of 93°C for 30 s, 55°C for 30 s and 68°C (Kogan protocol) or 72°C (Perkin-Elmer protocol) for 12 min in a thermal cycler (Omnigene-Temperature Cycler; Hybaid, Teddington, UK).

The cDNA clone for HsBENE (Lautner-Rieske et al., 1995; GenBank accession No. U17077) was kindly provided by H.G. Zachau (Ludwig-Maximilians-University, Munich, Germany) and the cDNA for H09290/H09291 (Hillier et al., 1995; GenBank accession Nos. H09290 and H09291) was obtained from the WashU-Merck EST Project. Computer analysis and alignments of sequences were performed using the GCG software package (Wisconsin Open-VMS V.8.0; Madison, WI, USA).

2.2. RNA isolation and Northern blot analysis

RNA was isolated from various organs of adult mice using the guanidinium-isothiocyanate method (TRIzol

kit: Gibco BRL) and analyzed by Northern blotting. The expression of the human cDNAs HsBENE and H09290/H09291 were analyzed on commercially available human tissue Northern blots (Clontech, Palo Alto, CA, USA). An α-actin probe was used to judge the uniformity of the human Northern blots.

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agcgtgtccagtcccaaggccgacgccagctcgcaggc ATG GCT CCG GCA GCG GCT TCG GGT GGC AGC  68
                                        M   A   P   A   A   A   S   G   G   S   10

ACC CTG CCC AGT GGC TTC TCG GTC TTC ACC ACC TTC CCT GAC TTG CTC TTC GTT TGT GAG  128
 T   L   P   S   G   F   S   V   F   T   T   F   P   D   L   L   F   V   C   E   30

TTT GTC TTT GGA GGC CTG GTG TGG ATC CTG ATT GCC TCC TCC CTG GTA CCC TTG CCC CTG  188
 F   V   F   G   G   L   V   W   I   L   I   A   S   S   L   V   P   L   P   L   50

GCC CAG GGC TGG GTG ATG TTT GTG TCT GTG TTC TGC TTT GTG GCC ACC ACT TCC CTG ATG  248
 A   Q   G   W   V   M   F   V   S   V   F   C   F   V   A   T   T   S   L   M   70

ATC TTG TAC ATA ATT GGT ACT CAT GGC GGT GAG ACT TCC TGG ATC ACA CTG GAT GCA GCC  308
 I   L   Y   I   I   G   T   H   G   G   E   T   S   W   I   T   L   D   A   A   90

TAC CAC TGT GTG GCT GCC CTA TTT TAC CTC AGT GCC TCA GTT CTG GAA GCC CTG GCC ACC  368
 Y   H   C   V   A   A   L   F   Y   L   S   A   S   V   L   E   A   L   A   T  110

ATC TCA ATG TTT GAT GGC TTT ACT TAC AAG CAT TAC CAT GAA AAC ATC GCC GCA GTG GTG  428
 I   S   M   F   D   G   F   T   Y   K   H   Y   H   E   N   I   A   A   V   V  130

TTT GCC TAC GTG GTC ACT CTG ATC TAC GTG GTC CAT GCT GTG TTT TCC TTA ATC AGA TGG  488
 F   A   Y   V   V   T   L   I   Y   V   V   H   A   V   F   S   L   I   R   W  150

AAG TCT TCA TAG gacagcagatcgggagctgagacccagatgcaattaactggtcagcccatcttccccattaact 564
 K   S   S                                                                    153

tcctagaacacagactgatggtggagaaaaagaaaacaagccaaaaagaaaacaaaacaaacacaaaaacaaaaggaagc 644
catattcaaccatattcgcgtctcttggggtgttatgtttaccttctgtcaaggtttagggcttgctatatttaaccttc 724
tagctaagggaggaaggagttgtcttggaaggggaccttttgcccttgaccgggacagttggtggggaactgggaacct 804
tgatctgagaatgacgatttacacttaccctgaatgtatgtcctaagaatttgcctcttggaatttttaagggctcttt 884
tggagcactcatcccctggatgtcttagtctttgtgaatagtctgggtgtcacaggcactgaaattcacccaacagaggt 964
ctccggacgttcacagtggaagatgagctcctttgaaatgcttcattaaatacttttgtgtctcatgcttcaggtgggga 1044
actggagggatgtctttcattcttgaggaccagggacaccttcctgttcaactttcttcctgcagagtcctttgattaac 1124
ctgattctcaagggacagttcagatgtgtgtgctgggccagtctttcccaaagtgactcactgggacgccaccctcacaa 1204
cagtgtccaccaggatagcgtctcccttgtagacacatcccgtagagctctgcctttgtctttcaacacaacagtctgct 1284
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tgtctgcagtctcgtgatgtgtttggacattgttctcagaccactccttgtgggagaaagggacctactctacagcaaag 1444
tgagaggtaatggctatctctggactgccacactccaggtgagggatgtctgacctgaaagaagaaagtataattaggaa 1524
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cactagctgatgttttattctccaacaaagtaggagattgagacctacaactaaattagatttggggatttttctatgtt 1764
ggttctcaggatggaacccaggggtttgcatgctgggtaagcactcttctaccagatcccatcatcagccccaaattgg 1844
atctcactggcacattcagaaggactttggttatcccagccatactggtatctgaattcaccatttgcccacttaacaca 1924
gccaaaatttcatactaaaacacaagcaaaagattaggggagttatttttaaaaattaaaatataaactaagagattat 2004
acaaagtttattattatgtttaaccaaaatctgtaactgagtgctgactggggtagtcttatactttcaacaaagctgaa 2084
ccctaacattacactaaccagcagctcaacacgagtgagatcacttttagacaagaagaagaaagagatgaaggaaggaa 2164
gaaaagagggaaaaggaagggtgactaagaggaattgcccaatttctcaatgtttcagattttaatctttaaaagaaac 2244
tttcatacaaaaaaaaaaaaaa                                                         2266
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Fig. 1. Sequence of the cDNA of mMAL. The mMAL transcript contains a 459 bp long open reading frame (indicated by capital letters) which is flanked by a 38 bp long 5'- and a 1775 bp long 3'-noncoding region (sequence submitted to the EMBL nucleic acid database: accession No. Y07626). The aa translation (indicated by the single letter code) predicts a protein of 153 aa with a calculated molecular weight of 16.6 kDa. No consensus sites for potential N-linked glycosylation are present.

A	RnMAL	CfMAL	HsMAL	Hsbene	H09290	RnPlas
MmMAL	91	85	87	36	37	29
RnMAL		87	89	40	38	27
CfMAL			88	38	37	24
HsMAL				41	38	26
Hsbene					42	28
H09290						25

B

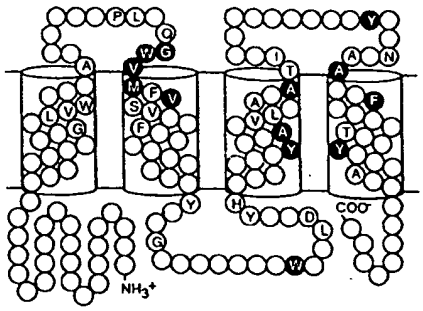


Fig. 2. Quantitative comparison of aa identities and a hypothetical model of the members of the MAL family. (A) Numerical comparison of the members of the MAL family indicates the uncorrected percentage of aa identities as calculated using the Distances program (GCG software package). (B) Schematic representation of the mouse MAL protein indicating the domains conserved within the MAL family. Residues conserved between at least six (out of the seven) members of the MAL family and their known species homologues are lettered. Those residues conserved in all members are indicated by filled circles.

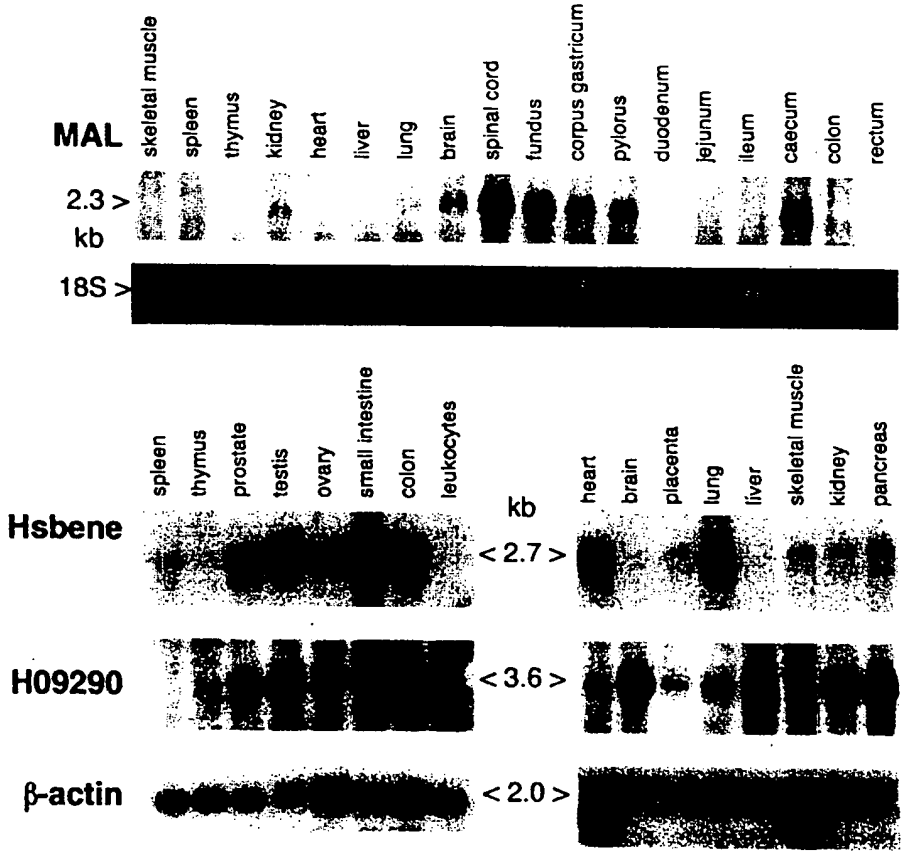
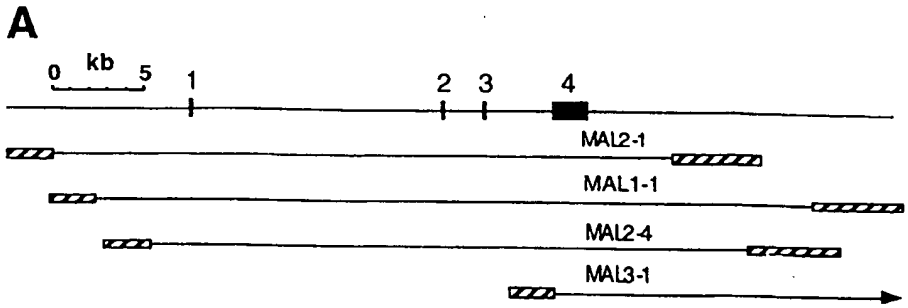


Fig. 3. Northern blot analysis of mMAL, BENE and H09290 expression. mMAL mRNA levels were examined in different tissues of 3-month-old mice. Ethidium bromide staining is shown as loading control. Analogously, the expression of HsBENE (2nd panel) and H09290 (3rd panel) were analyzed using human RNA. Estimated molecular weights of the detected transcripts are indicated. As control, the signal obtained by hybridization of the same membrane with a β-actin probe is shown. A unique 1.8 kb band in heart and skeletal muscle tissues due to cross-hybridization with α-actin is also observed.



B

exon number size	exon	intron size	exon
exon 1 >131bp	...TGT GAG TTT ...cys glu phe 31	gtgagtgata.. ..cctcctgcag ≈14 kb	GTC TTT GGA... val phe gly... 32
exon 2 168 bp	...ATC ACA CTG ...ile thr leu 87	gtgagtctgg.. ..tcacccacag ≈1.7 kb	GAT GCA GCC... asp ala ala... 88
exon 3 126 bp	...GCC GCA GTG ...ala ala val 129	gtaagttccc.. ..ctggttccag ≈3.8 kb	GTG TTT GCC... val phe ala... 130
exon4 >1841 bp			

Fig. 4. Schematic structure of the mMAL gene. (A) The structure of the mMAL gene consists of four exons (filled boxes) and three introns (indicated by a thin line). The isolated and characterized clones with flanking cosmid sequences (indicated as striped boxes) are shown. (B) Exon-intron boundaries of the mMAL gene were determined by direct sequencing of the cosmid clones (sequences were submitted to the EMBL nucleic acid sequence database; accession Nos. Y07627–Y07630). Exon sequences are in capital letters with the aa translation indicated below.

3. Results and discussion

3.1. Cloning and analysis of the mMAL cDNA

The mMAL cDNA was cloned from an 8-day-old mouse brain cDNA library using the rMAL cDNA as a probe. Ten positive clones were isolated by screening approx. 5×10^5 phage plaques. Two clones carried full-length mMAL cDNAs containing the entire coding region of mMAL as well as a short 5′-untranslated region and a relatively long 3′-untranslated stretch (Fig. 1). Comparison of the mMAL cDNA to its known species homologues revealed an overall nucleic acid identity of 86% to rMAL (Schaeren-Wiemers et al., 1995b), 71% to hMAL (Alonso and Weissman, 1987) and 68% to cMAL (Zacchetti et al., 1995). If only the coding regions are included in the comparative analysis, the nucleic acid identities are further increased to 93% (rMAL), 86% (hMAL) and 84% (cMAL). The corresponding interspecies aa identity values range from 91% (rMAL) to 85% (cMAL; Fig. 2A).

Computer-aided databank searches using the mMAL aa sequence as a probe detected several related cDNAs which appear to define a novel gene family. These cDNAs include human HsBENE (Lautner-Rieske et al., 1995), the human expressed sequence tag (EST) H09290 and rat plasmolipin (Fischer and Sapirstein, 1994; data not shown). Hydrophobicity plots suggest that MAL, HsBENE and plasmolipin have similar predicted structures with four potential transmembrane domains, while the EST cDNA clone H09290 is not full length and the putative first transmembrane domain near the amino terminus is missing in the predicted protein (data not shown). Intraspecies comparisons of aa sequences identified HsBENE and H09290 as close relatives to hMAL (aa identities of 41% and 38%, respectively; Fig. 2A). In contrast, the overall aa identity of 27% shared by rat plasmolipin and rMAL is less significant in this type of analysis (Fig. 2A). However, the alignment of a generic MAL consensus sequence obtained from the comparison of MAL species homologues (mouse, rat, human and canine) reveals that plasmolipin is likely to be a distant member of the MAL family (data not shown). In particular, the aa sequence -(Q,Y)GWVM(F,Y)V(S,A)(V,L)- which is located at the presumed junction of the first extracellular loop and the second transmembrane domain appears to be a common denominator (Fig. 2B). Database searches using this aa motif showed that it is exclusively found in members of the MAL protein family (data not shown). The likely structural

and/or functional importance of this motif which is characteristic for this protein family remains to be determined.

3.2. Analysis of mRNA expression

Northern blot analysis was performed to assess the expression patterns of the different members of the MAL family (Fig. 3). In agreement with the results obtained in the rat (Kim et al., 1995; Schaeren-Wiemers et al., 1995b), mMAL is expressed in the spinal cord, brain and kidney. In addition, we found prominent expression of mMAL in the gastrointestinal tract, in particular in the stomach (fundus, pylorus and corpus gastricum) and in part of the large intestine (caecum; Fig. 3).

Similar to mMAL, H09290 is also highly expressed in the brain and the kidney. Furthermore, significant levels of H09290 mRNA are found in the prostate, testis, intestine, heart, placenta, lung, liver and pancreas (Fig. 3). In contrast to mMAL and H09290, HsBENE mRNA is not found in the brain but it is mainly expressed in prostate, testis, gastrointestinal tract, heart and lung (Fig. 3).

3.3. Cloning and analysis of the mMAL gene

The mMAL cDNA was used to screen a mouse genomic cosmid library. Four positive clones were isolated from approx. 9×10^5 colonies (clones 1-1, 2-1, 2-4 and 3-1; Fig. 4A) and subjected to restriction mapping and Southern blot analysis (data not shown). Clones 2-4, 1-1 and 2-1 were found to include 5′-noncoding regions of approx. 2 kb, 5 kb and 8 kb, respectively (Fig. 4A and data not shown).

The sizes of introns 1 (approx. 14 kb), 2 (1.7 kb) and 3 (3.8 kb) were determined by long-range PCR, and the splice acceptor and donor sites of all introns were identified by direct DNA sequencing. All exon-intron boundaries were shown to be in agreement with the -GT...(5′ donor)...AG- (3′ acceptor site) rule (Fig. 4B).

4. Conclusions

(1) MAL, plasmolipin, BENE and the EST clone H09290 define a novel gene family which is widely expressed in various mammalian tissues suggesting a basic role of these related molecules in cell biology.
(2) The members of the MAL/plasmolipin/BENE H09290 family share 29–37% aa identities and have similar predicted structures as tetraspan membrane proteins.
(3) The shared aa sequence -(Q,Y)GWVM(F,Y)V(S,A)(V,L)- which is uniquely found in this protein family may serve as a molecular fingerprint for the identification of members of this family.

Acknowledgement

We thank Corinne Zgraggen for excellent technical assistance and Christian Lobsiger and Dr. Verdon Taylor for providing RNA blots. This work was supported by a grant of the Swiss National Science Foundation to U.S.

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